Cellular immune controls over Epstein-Barr virus infection: new lessons from the clinic and the laboratory

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Abstract: Epstein-Barr virus (EBV), a human herpesvirus with potent B cell growth transforming ability, induces multiple cellular immune responses in the infected host. How these host responses work together to prevent virus pathogenicity, and how immune imbalance predisposes to disease, remain poorly understood. Here, we describe three ongoing lines of enquiry that are shedding new light on these issues. These focus on: (i) patients with infectious mononucleosis or its fatal equivalent, X-linked lymphoproliferative disease; (ii) EBV infection in a range of new, genetically defined, primary immune deficiency states; and (iii) experimental infection in two complementary animal models, the rhesus macaque and the human haemopoietic stem cell reconstituted mouse.

DOI: https://doi.org/10.1016/j.it.2014.01.003

Cellular immune controls over Epstein-Barr virus infection: new lessons from the clinic and the lab

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Biology of EBV infection : an overview

Herpesviruses are ancient pathogens whose apparently benign relationship with their host species reflects the fine balance struck between host immune controls over virus infection and the virus' ability to evade those controls. Real insight into the immunological battle raging beneath the surface comes when that evolutionary compact is disturbed and disease ensues. Nowhere is this better illustrated than by Epstein-Barr virus (EBV), the human gamma-1 herpesvirus carried by most people as a life-long asymptomatic infection. Figure 1 presents the basic framework of EBV infection as currently understood. The main features, in chronologic order, are (i) initial replication of orally transmitted virus in permissive cells (probably squamous epithelial cells and some locally-infiltrating B lymphocytes) within the oropharynx,, (ii) colonisation of the host through growth-transforming latent infection of B cells in oropharyngeal lymphoid tissues, (iii) life-long persistence within the re-circulating memory B cell pool as a silent latent infection, and (iv) occasional reactivation from latency into virus replicative ("lytic") cycle, seeding secondary foci of virus replication at oropharyngeal sites.

In the process the virus presents an antigenically rich challenge to the immune system. Thus virus replication involves the sequential expression of two immediate early, >30 early and >30 late lytic cycle proteins, while classical B cell growth-transformation is achieved through the collective actions of 8 latent proteins, namely the nuclear antigens EBNAs 1, 2, 3A, 3B, 3C and –LP plus the latent membrane proteins, LMPs1 and 2. Many of these viral proteins elicit antibody and/or cell-mediated immune responses. Among the humoral responses, neutralising antibodies, predominantly directed against the virus' major envelope glycoprotein gp340 (a late lytic cycle product), are almost certainly important in longer-term protection of the host from re-infection by exogenous virus; however, such antibodies are quite slow to develop to high affinity during primary infection. Accordingly, cell-mediated responses appear to be more important in bringing the primary infection under control and also in containing reactivation of the virus from its latent reservoir during long-term virus carriage.

The main effectors of these cellular responses, NK cells, $CD4^+$ and $CD8^+$ T cells, are superimposed onto Figure 1 to show their potential sites of action against infected cells. How

important these individual players are, and how they work together to contain EBV as an asymptomatic infection, is still poorly understood. Here we review recent lessons on this subject that have come either from patients in whom natural infection erupts into disease or from experimental infection in two increasingly promising animal models.

Infectious mononucleosis

Primary EBV infection in its natural setting, early in life, is almost always asymptomatic. However, if delayed until adolescence or later, as often happens in affluent societies, it sometimes presents as infectious mononucleosis (IM). This disease is characterized by large expansions of activated $CD8^+$ T cells and to some extent NK cells appearing in the blood; indeed the classical symptoms (fever, pharyngitis, lymphadenopathy) may be caused more by cytokine release from those activated cells than from the virus infection *per se*. However the course of IM can vary from mild to severe, and little is known about the risk factors for disease development or severity. The most recent insights have come from a 4-year follow-up of 143 EBV-seronegative college students, involving regular screening at 8 week intervals and immediate bleeds on signs of illness [1]. Of the 66/143 students who seroconverted to EBV-positivity over that time, 51 developed two or more IM symptoms. Disease severity correlated positively with virus load in circulating B cells, with the size of the $CD8^+$ T cell response and also with NK cell numbers in the blood. Although these findings could not distinguish between virus infection and immune pathology as the determinant of symptoms, they did suggest that both $CD8^+$ T cells and NK cells were responding similarly to the intensity of the viral challenge. The latter point remains controversial, however, since earlier work (albeit on a smaller patient cohort) reported an inverse correlation between virus load and NK cell numbers in IM blood, and inferred from this that severe IM resulted from a deficiency of early NK-mediated control [2]. Further studies are needed to resolve this important issue, focusing on the phenotype as well as the number of NK cells in IM blood, and (in the rare cases where this is possible) extending the work to include an analysis of NK cell, $CD8^+$ T cell and EBV-infected B cell populations in IM tonsils, where the real action is likely to be found.

As comparators with IM, cases of asymptomatic primary infections are potentially very informative but they are difficult to identify without regular screening of large EBV-naïve cohorts. The above prospective study of college students [1] did not report on this topic but recent work in young children, albeit in Africa, found that viral loads in the blood can be as high as in acute IM yet they do not induce obvious lymphocytosis [[3], *Jayasooriya and Hislop, personal comm*]. This is reminiscent of earlier findings, where two of three asymptomatic seroconverters identified during screening for a vaccine trial had high EBV loads without large $CD8^+$ T cell expansions [**Silins et al 2001**]. Such evidence favours the view of IM as an immunopathologic disease, but why CD8 expansion should be more common after acquiring the

virus later in life is not understood. Virus load *per se* cannot be the full story. One suggestion is that adults are primed to mount more vigorous $CD8^+$ T cell responses because, with age, other viral challenges have populated T cell memory with more clonotypes cross-reactive with EBV [4]; so far however, prospective studies show no clear evidence of pre-existing $CD8^+$ T cell responses to other viruses being expanded (or eroded) in IM [5]. Another possibility is that NK cell responses assume greater importance, and perhaps are more effective, in combating virus infections early in life [6]; this again accords with the idea that effective early control over the incoming virus by NK cells can obviate the later need for massive CD8 expansion and its associated symptomatology.

Components of the cell-mediated response to EBV

NK and invariant (i-)NKT cell responses

NK cell control of EBV during primary infection may operate at several levels, targeting either virus replicative foci in the oropharynx or the latent growth-transforming infections through which the virus first colonises the B cell system (see Figure 1). Of the two, virus replication would seem the more likely target if only because expression of surface HLA I molecules, the ligands for NK inhibitory receptors, falls in lytically-infected cells. In vitro models of lytic infection are limited but early studies showed that, at least in a virus-infected Burkitt's lymphoma cell line, the subset of cells entering lytic cycle are indeed sensitised to NK recognition [7]. This was coincident not just with surface HLA I down-regulation but also with up-regulation of ligands for the NK activating receptors NKG2D and DNAM-1 [7]. If NK cells have indeed exerted evolutionary pressure on EBV, one might expect viral counter-measures against such recognition, and perhaps the first hint of such NK evasion has recently emerged. Thus one of the EBV-coded lytic proteins that impairs HLA class I antigen presentation to $CD8^+$ T cells at the cell surface, BILF1, targets HLA-A and -B molecules but not HLA-C, the ligand for certain NK inhibitory receptors of the KIR2DL class [8]. Further insights into the signals mediating NK recognition of lytically-infected cells are long overdue.

As to latent infection, EBV-transformed LCLs are poorly recognised and killed by the cytotoxic $CD56^{dim}$, $CD16^+$ NK cells that are prevalent in blood, probably reflecting the dominance of inhibitory over activating signals mediated by the high HLA I levels seen on transformed B cells [**Stewart et al, PNAS 2005**]. However in secondary lymphoid organs, including the tonsils where EBV infection initiates, the NK cell population is dominated by less mature $CD56^{bright}$, $CD16^-$ cells, some of which are primed for rapid IFNγ production in response to dendritic cell-derived IL12 rather than cytotoxicity [9]. The superior ability of tonsillar, as opposed to blood, NK cells to limit B cell transformation by EBV in vitro [10] has now been attributed to this high-IFNγ-producing $CD56^{bright}$, CD16- subset [11]. Accordingly, this effect requires the addition of NK effectors to the cultures within 2-3 days of B cell infection, that is during a brief initial

period in which the B cell transformation process can likewise be delayed (but never ablated) by exogenous IFNγ addition.

Recent evidence suggests that iNKT cells with the Vγ9/Vδ2 T cell receptor, recognising the α-GalCer-CD1d complex, may also mediate some control over the same in vitro transformation process. Thus, although iNKT cell numbers in peripheral blood mononuclear cell (PBMC) preparations are typically <0.3%, their depletion using an α-GalCer-CD1d tetramer reportedly led to a 2-fold increase in the number of activated EBV-infected B cells in PBMC cultures 8 days post-infection [12]. The mechanism underlying this effect remains to be determined, but presumably must operate within 1-2 days of infection before EBV-activated B cells down-regulate CD1d expression. Importantly, this down-regulation is not evidence of iNKT cell evasion by the virus but the programmed response of resting human B cells to any activating signal [13].

$CD8^+$ T cell responses

The cytotoxic $CD8^+$ T cell response to primary EBV infection as seen in IM patients has been extensively reviewed [14,15] and only certain aspects will be discussed here. Most activated $CD8^+$ T cells in IM blood are EBV-specific, with lytic-antigen dominant over latent-antigen specificities. This dominance becomes less marked as the acute infection subsides and the highly expanded lytic-antigen response is more heavily culled. These changes, graphed in Figure 2, are consistent with the presumed order of events in primary EBV infection. Early replicative foci would be expected to activate responses first from NK cells and then from lytic antigen-specific $CD8^+$ T cells; thereafter, the virus colonises the B cell pool through latent growth-transforming infections that elicit the latent antigen-specific CD8 response.

The latent cycle proteins are co-expressed in virus-transformed cells yet, as CD8 targets, show a marked hierarchy of immunodominance with the strongest responses on many HLA I backgrounds recognising epitopes derived from the largest latent cycle proteins, the transcriptional regulators EBNAs 3A, 3B and 3C [15]. However there are exceptions; particular HLA alleles will induce strong responses against epitopes from one of the usually sub-dominant antigens, for example the virus genome maintenance protein EBNA1 [16]. Thus endogenously expressed EBNA1 can be a significant CD8 target despite impairment of its HLA I-restricted presentation by the protein's glycine-alanine repeat (GAr) domain; this reflects the fact that the impairment is only partial and does not eliminate EBNA1 epitope display [**Mackay et al, 2009**]. Though outside the scope of the present review, readers are referred to papers attributing the GAr domain's action either to its mRNA structure slowing translation [17,18] or to the nascent GAr polypeptide itself impairing translation initiation [19,20] .

Lytic cycle antigens also display a reproducible hierarchy of immunodominance in donors across a range of HLA types, with the numerically strongest responses directed against the two immediate early and a subset of early proteins, and much weaker responses against the virion structural proteins that are abundantly expressed late in the cycle [21,22]. Interestingly, this hierarchy directly matches the falling efficiency with which these successively expressed antigens are presented on the lytically-infected cell surface [21], as EBV's battery of CD8 evasion proteins take increasing hold (reviewed [23,24]). This correlation between immunogenicity and epitope display on infected cells implies either that the $CD8^+$ T cell response is initially primed by direct contact with lytically-infected B cells (rather than cross-primed by antigen re-presented in dendritic cells) or, at the least, its subsequent expansion is directly driven by such contacts. The arguments favouring direct B cell contact as the main driver of EBV-specific $CD8^+$ T cell responses are enlarged later (see Figure 3 legend) when looking at the possible consequences for antigen choice in individuals with impaired B cell/T cell interactions.

$CD4^+$ T cell responses

By contrast, the $CD4^+$ T cell response to EBV seems to be spread much more evenly across the whole range of available lytic cycle as well as latent antigens, as one might expect from a classically cross-primed response [25,26]. There is no marked increase in total $CD4^+$ T cell counts in acute IM but, with the development of HLA II tetramers, activated EBV epitope-specific $CD4^+$ T cells have been detected at this time [27]. While these individual epitope-specific responses rarely exceed 1% of all $CD4^+$ T cells, collectively they do impact on the activation status of the circulating $CD4^+$ population in the acute phase. Thereafter tetramer-staining cell numbers fall rapidly within a few days, suggesting that the response peaks very early in the disease course. Again in contrast to the CD8 response, latent antigen-specific CD4 responses tend to be larger than those directed against lytic antigens both in IM and, after contraction, in T cell memory [27]. It is notable that, at least after in vitro expansion, many EBV-specific $CD4^+$ T cell clones have cytotoxic as well as cytokine-secreting abilities [26,27]. This implies that the $CD4^+$ T cell response may play more than one role in vivo, with some cells providing help for the antibody responses to EBV antigens typically seen in IM, and others acting as direct cytolytic effectors against virus-infected B cells.

Intriguingly, the primary CD4 response to EBNA1 displays quite different kinetics, often not appearing until months after disease resolution [27]. This matches the well-known serologic picture in IM, where the IgG response to EBNA1 is unusually delayed and indeed may never appear in rare patients with chronic IM-like symptoms [28-30]. An absence of specific T cell help might therefore underlie this previously unexplained feature of the EBNA1 antibody response. As to why the EBNA1-induced T cell response is so delayed, a clue came from studies

looking at the recognition of LCLs by $CD4^+$ T cell clones specific for EBV antigens naturally expressed by these cells. Clones specific for lytic cycle antigens and many clones against latent proteins recognised LCLs well. In each case, however, this did not reflect direct recognition of antigen-expressing cells; rather, the relevant antigen was being released into supernatant medium, then taken up and processed as exogenously-acquired protein by co-resident cells in the culture. By contrast, EBNA1 was not detectably released and so its presentation to $CD4^+$ T cells was entirely dependent on intra-cellular processing of the endogenously expressed protein [31,32]. This perhaps explains why only a minority of the known EBNA1 CD4 epitopes are displayed on the LCL surface in amounts that are detectable by epitope-specific T cell clones, and those that are presented arise through intra-cellular processing pathways such as macro-autophagy [32,33].

Figure 2 summarises a kinetic view of events characterising primary infection, from the time IM symptoms develop. Note that very little is known about the prior incubation period, although some evidence suggests that high virus load in the blood and the accompanying lymphocytosis only appear shortly before symptoms [1]. Interestingly, while latent virus load in the blood is rapidly brought under control during the disease course , high levels of asymptomatic virus shedding can be found in throat washings for several weeks or months [30,34]. This suggests either that shedding is occurring from an immunologically protected site in the oropharynx or that the particular controls governing shedding are slow to develop and access that site.

EBV infection in primary immunodeficiencies affecting cellular immunity

While classical IM patients offer one important window into the biology of EBV infection, patients with primary immunodeficiencies affecting the NK and/or T cell systems offer another. The best known examples, the X-linked lymphoproliferative syndrome (XLP) and its relative XIAP, have recently been joined by several other primary immunodeficiencies predisposing to EBV-associated disease [reviewed [35]]. These are of considerable interest but require cautious interpretation because patient numbers are small and, even where the genetic basis of the deficiency is known, its effects are rarely specific to just one arm of the cellular immune system.

There are currently three genetically-defined conditions with either a selective or preferential loss of NK cell function relative to that of T cells [reviewed [36]]. All three are associated with increased susceptibility to various pathogens, including herpesviruses such as herpes simplex (HSV1) and varicella (VZV); however EBV is also implicated on occasions. The most NK-specific deficiency involves patients with a homozygous mis-sense mutation in CD16, the NK cell activating receptor for antibody-dependent cell cytotoxicity (ADCC). Interestingly, the mutation does not affect NK cell development or ADCC function but does impair spontaneous NK-mediated cytolysis [37-39]; among three affected kindreds, there was one case of prolonged IM-like illness in infancy and another case of EBV-driven lymphadenopathy. Another condition

affecting, among other things, NK but not T cell development arises from homozygous mutation of the gene encoding MCM4, a helicase component of the DNA replication complex [40-42]. Among the few MCM4-deficient kindreds studied to date, one child developed EBV-positive B-lymphoproliferative disease (i.e. the in vivo outgrowth of virus-transformed cells, just as occurs in T cell-suppressed transplant patients); interestingly this child was the only one of four siblings who also failed to mount a T cell-dependent EBNA1 antibody response to EBV infection [40]. Finally an overlapping set of clinical presentations, for which an NK cell deficiency with susceptibility to herpesvirus infections [43] represents the index case [44], have recently been linked to heterozygous mutations of the haemopoietic transcription factor GATA2 [45]. Though the haematological picture is varied, most affected individuals show severe depletion of circulating NK cells, B cells and monocytes and a partial loss of $CD4^+$ T cells. Interestingly, perhaps because an absence of B cells would greatly reduce the chance of acquiring EBV, there were only two cases of EBV-associated disease among the 57 patients identified to date and both involved unusual EBV-positive malignancies of mesenchymal cells [45]. One was of spindle cell origin whereas, significantly, the other was a leiomyosarcoma, a smooth muscle cell tumour usually observed only in heavily T cell-compromised patients [46,47] but recently also found in a child with a genetically-uncharacterised NK cell deficiency [48].

Clearly NK cell defects impair host control over the replication of several viruses, including such agents as HSV1 and VZV. The same may well be true for EBV but this is less clinically obvious because, in contrast to other herpesviruses, uncontrolled EBV replication in its natural setting of the oropharynx is either asymptomatic or manifest as non-threatening oral lesions [49]. To what extent NK cells have a restraining role over growth-transforming latent EBV infections is less clear from the above evidence. The appearance of rare EBV-positive tumours derived from atypical cell lineages may well reflect the fact that latent EBV infection is potentially oncogenic in many cellular contexts, but that the virus only gains opportunistic access to these other cell lineages when virus replication is poorly controlled.

Three other recently-identified immune deficiencies associated with symptomatic EBV infection share a broadly similar immunologic phenotype with normal NK cell numbers, low iNKT cell numbers (where examined), and marked T cell lymphopenia, especially affecting naive $CD4^+$ T cells. These involve homozygous mutations in the genes encoding (i) interleukin2-inducible T cell kinase (ITK), a non-receptor tyrosine kinase involved in TCR signalling but also important for iNKT cell development [50-52], (ii) coronin1A, an actin regulator involved in TCR signalling and T cell homeostasis [53], and (iii) serine-threonine kinase 4 (STK4), with roles in T cell homing and function [54,55]. Though many such individuals have a history of recurrent viral infections, most have been investigated specifically because of a clear predisposition to EBV-driven B-lymphoproliferative disease and/or EBV-positive Hodgkin Lymphoma. There was no detailed analysis of EBV-specific T cell responses in the above patient cohorts, but the findings chime with a wealth of existing evidence from transplant patients receiving T cell-suppressive

drugs [reviewed [56]] that T cells exercise the main control over growth-transforming B cell infections in vivo.

However, as more primary immunodeficiencies are genetically mapped and their cohorts investigated, three other conditions have been recognised with impaired EBV control despite the induction of a sizeable $CD8^+$ T cell response. The first is caused by a dominant-activating mutation in the catalytic subunit of the phosphatidylinositol-3-OH kinase (PI3K) pathway, leading to lymphocyte hyper-activation [57,58]. This typically presents in childhood as susceptibility to respiratory infections but, in one report, all 9 affected patients had high EBV genome loads in the blood and 2 had developed EBV-positive lymphoma. Patients had large numbers of EBV-specific $CD8^+$T cells; however, like the $CD8^+$ T cell population as a whole, these were skewed toward a terminally differentiated, potentially functionally impaired, phenotype [58].

A second such condition involves homologous inactivating mutations in the gene encoding CD27, a TNF receptor family member expressed by memory B cells and by all T cells except for a late terminally differentiated subset. Perhaps surprisingly, CD27 loss did not affect memory B cell development or NK, i-NKT, $CD4^+$ and $CD8^+$ T cell numbers. However, 8 of 10 affected members in three kindreds presented either with severe primary EBV infections, often leading to hypogammaglobuinaemia, or with EBV-positive B-lymphoproliferative disease [59,60]. Surviving patients in both studies had EBV-specific $CD8^+$ memory T cell populations that were of normal size but markedly skewed towards an IFNγ-producing (effector-memory) phenotype, possibly reflecting on-going antigen challenge in vivo. One interesting possibility is that these patients are particularly susceptible to EBV because virus-transformation strongly upregulates the CD27 ligand, CD70, on the B cell surface [61], and optimal recognition by EBV-specific CD8+ T cells may require the CD27-CD70 interaction as a co-stimulus.

A final example involves inactivating mutation of the X-linked gene encoding the magnesium transporter, MAGT1 [62]. Affected males have normal NK and i-NKT cell numbers but low $CD4^+$ T cell counts, especially in the naive compartment, and a TCR signalling defect [63]. Though susceptible to other infections, all 7 reported patients came to attention through persistent IM-like symptoms and high EBV loads, progressing in 4 cases to EBV-positive B-lymphoproliferative disease or frank lymphoma. These patients had normal numbers of EBV-specific $CD8^+$ memory T cells and NK cells; however, after in vitro expansion, both these effectors showed poor recognition of relevant target cell lines. Mg^{++}-supplementation of the medium corrected the functional deficit by restoring expression of NKG2D both on the NK cells, where it serves as an activating receptor, and on the EBV-specific $CD8^+$ T cells, where interactions with NKG2D ligands on the LCL surface optimised specific recognition. Remarkably, treating the MAGT1-deficient patients with Mg^{++} supplements led to falling EBV loads in the blood, demonstrating the biological relevance of NK and/or T cell responses in setting the viral set-point in vivo [64].

The special case of XLP and its relative XIAP

The most extreme example of genetic susceptibility to EBV involves young boys in whom primary infection leads to X-linked lymphoproliferative disease (XLP), a highly exaggerated form of IM, with high viral loads in infected B cells accompanied by massive $CD8^+$ T cell and NK cell responses [65]. The resultant cytokine storm triggers haemophagocytic lymphohistiocytosis (HLH), leading in most cases to fatal bone marrow failure. Other boys with the same trait present with hypogamma-globulinaemia or B cell lymphoma, conditions that are not linked to (and may precede) EBV infection [66-68]. The XLP gene *SH2D1A* encodes a small adapter protein, SAP, that is expressed in T, NK and i-NKT cells and is involved in the signalling of cell-cell interactions mediated by members of the SLAM family of surface receptors [69] Interestingly, XLP patients have normal numbers of NK and T cells but lack i-NKT cells, fuelling speculation that i-NKT cells may play a role in controlling EBV. However, as discussed further below, SAP-negative T and NK cells also have specific functional defects that likely contribute to the disease.

Subsequently, a second X-linked condition with similarities to XLP was discovered and mapped to the nearby *BIRC4* gene [70]. Its product, the X-linked inhibitor of apoptosis (XIAP) protein, is ubiquitously expressed and has both an anti-apoptotic function and multiple signalling pathway connections. Affected boys typically present with HLH symptoms that are often, but not always, linked to primary EBV infection; they may also present with hypogamma-globulinaemia but, in contrast to XLP, never with lymphoma. Just how close the relationship is between the two conditions remains a subject of debate [71,72]. XIAP patients often have low numbers of i-NKT cells but normal NK and T cell numbers; however their T cells show an increased sensitivity to apoptotic stimuli ex vivo [70,73]. How this immune phenotype relates to the XIAP disease phenotype is still poorly understood.

By contrast, work on SAP has illuminated many aspects of XLP pathogenesis. SAP binds to the cytoplasmic tails of SLAM family proteins expressed by NK, T and i-NKT cells and mediates signal transmission following the interaction of these proteins with their partners on other cell types. Interactions with B cells appear particularly dependent on two SLAM family members; one of these, NTBA, forms homotypic interactions with NTBA on the B cell surface whereas the other, CD244, binds to a B cell-specific protein, CD48, whose expression is dramatically up-regulated by EBV infection (see Figure 3). SAP deficiency is therefore especially damaging both to physiologic T cell-B cell communication, hence predisposing to hypogamma-globlinaemia [69], and to the control of a B-lymphotropic virus: thus NK cells cannot kill HLA class 1-negative B cell targets [74] and EBV-specific $CD8^+$ T cells, though they are induced in XLP patients, cannot recognise EBV-infected B cell targets [75]. The same deficient target recognition also protects XLP T cells from restimulation-induced cell death, the homeostatic

control that normally regulates effector cell numbers [76], hence explaining the extreme expansions and associated symptomatology one sees in the fatal IM-like disease.

The importance of SAP for $CD8^+$ T cell control of EBV is most clearly shown by XLP-carrier mothers, in whom random X-chromosome inactivation produces both SAP-positive and SAP-negative populations in the T cell repertoire. EBV-specific $CD8^+$ T cells in these carriers were exclusively SAP positive, while $CD8^+$ T cells against other viruses were found in both populations [77]. Furthermore, careful re-analysis of XLP patients themselves has detected cases where small numbers of $CD8^+$ T cells have regained SAP function by somatic reversion of the *SH2D1A* gene mutation. Such SAP-positive cells were only detectable in XLP patients who had survived EBV infection and indeed those cells appeared to be enriched for EBV-reactivities, suggesting that long-term virus carriage had driven the expansion of these rare revertants to detectable levels [78].

Despite the presence of small reverted populations in most EBV-positive XLP patients studied to date, all such patients also have SAP-negative $CD8^+$ T cell memory to EBV. Hence they have clearly mounted $CD8^+$ T cell responses to primary EBV infection even though the SAP-negative effectors thus produced recognise EBV-infected B cells very poorly if at all [75,77]. The implications for the priming of these responses are illustrated in Figure 3. In XLP patients the initial priming and early expansion of the CD8 response might be driven less by direct $CD8^+$ T/B cell contact, that is by the pathway envisioned for responses in immunocompetent individuals, and more by the cross-presenting DC pathway. As a result, the usual hierarchy of CD8 immunodominance among lytic cycle proteins (immediate early>early>late, reflecting antigen display on lytically-infected B cells) should be overturned. In particular, late structural proteins of the virus, released in abundance from lytically-infected cells and available to cross-presenting DCs, should compete more effectively for immunodominance. Antigen choice would therefore become much broader, indeed closer to that shown by the conventionally cross-primed CD4 response. Screening the hierarchy of EBV antigen specificities within the SAP-negative $CD8^+$ T cell pool of XLP patients will allow this prediction to be tested.

Lessons from animal models

Lessons learned from EBV infections in the natural host can take us so far. However many outstanding issues, the very early events following infection of the naïve host, the establishment of latency in memory B cells, the relative contributions of innate and adaptive responses to immune surveillance, can only be resolved with the help of animal models. The restriction of gamma1-herpesvirus infections to primate hosts is a major challenge in that regard, but two models show particular promise.

Old World primates carry gamma1-herpesviruses that are genetically very close to EBV, persist in the B cell system and elicit both $CD4^+$ and $CD8^+$ antigen-specific T cell memory [79,80].

Furthermore the establishment of a specific pathogen-free colony of rhesus macaques has allowed such animals to be orally infected with their natural EBV-like agent, rhesus lymphocryptovirus (rhLCV) [81]. As recently reviewed [82], now that the rhLCV genome can be manipulated genetically as a Bacterial Artificial Chromosome, the system has the ability to identify what role individual virus genes play in the acute and persistent phase of infection. Indeed, a serendipitous deletion in the gene encoding rh-BARF1, a secreted lytic cycle protein that blocks colony stimulating factor (CSF1) signalling, has already produced interesting results. Rh-BARF1 evasion of the host's innate CSF1 response increases virus replication in the oropharynx, and this leads on to higher loads in latent virus in the life-long B cell reservoir [83].

A second complementary approach uses immunodeficient BALB/c Rag $2^{-/-}$ $\gamma c^{-/-}$ or NOD-scid $\gamma c^{-/-}$ mice reconstituted with the main human haemopoietic cell lineages from inocula of $CD34^+$ human stem cells from cord blood or foetal liver [reviewed [84]]. Initial studies showed that EBV infection of such animals by intraperitoneal injection induced virus-specific $CD8^+$ T cell responses and that T cell depletion predisposes to virus-driven B-lymphoproliferative disease [85-87] . Interestingly, although the virus had to be delivered directly into the B cell system of these animals (by-passing the stage of virus replication in the oropharyynx), there was a detectable $CD8^+$T cell responses to lytic cycle epitopes, confirming that incoming virus can replicate in B cells of the naïve host. Analysing human T cell responses to any virus in such animals is complicated by the need to supply a human HLA-expressing thymic epithelium for selection of the T cell repertoire, either via HLA transgenesis or using a human thymic tissue-containing organoid [86,88,89]. However, animals with successful reconstitution of the dendritic and NK cell compartments [90-92] allow one to study the very early immune response to virus infection, before T cells come into play. Very recent work in such animals has thrown important light on the early events of EBV infection [93], detecting marked expansion of NK cells with an early-differentiation $CD94^+NKG2A^+KIR^-$ phenotype. Note that this phenotype is either identical to or the next differentiation step after [94] the tonsillar NK cell compartment that showed superior inhibition of EBV driven B cell transformation in vitro [10,11]. Antibody-mediated depletion of these cells, but not of more differentiated NK cells, led to increased EBV genome load in blood and spleen (with especially high DNA-aemia in plasma) and increased expression of lytic EBV antigens in infected organs. This effect of NK cell depletion on viral load was only observed using wild-type virus, not with a replication-deficient EBV mutant, thereby clearly implicating a role for NK cells in controlling early virus replication. Furthermore NK cell depletion and the consequent increase in virus load led to a greatly expanded $CD8^+$ T cell response, associated with elevated cytokine levels and lymphadenopathy; again the effect was only observed with replication-competent wild-type virus. These findings provide the first experimental evidence, albeit in an animal model, that NK cells and T cells play important complementary roles in combating primary EBV infection. As illustrated in Figure 4, they suggest that NK cells, particularly the early-differentiated subset that predominates in early childhood, act initially to limit lytic EBV replication and thereby avoid the massive expansion of virus-specific $CD8^+$ T cells that culminates in IM-like immunopathology.

Conclusions and future challenges

EBV is a very significant human pathogen yet, in most individuals, is carried as a completely asymptomatic infection contained by host immune controls. Given the global impact of EBV-associated malignant and non-malignant conditions, understanding the full spectrum of those immune controls and how their disturbance can lead to disease is a hugely important goal. Here we have described three current lines of enquiry that will remain important in pursuing that goal: (i) prospective studies on IM and, where possible, on the exaggerated form of this disease seen in XLP patients, (ii) monitoring EBV infections in the ever-increasing range of genetically-defined primary immune deficiency states, and (iii) experimental EBV infections in the rhesus macaque and humanised mouse models. With these studies as a basis, there are many further questions to address. How might EBV infection, particularly a history of IM, predispose both to an autoimmune disease, multiple sclerosis [95] and to Hodgkin Lymphoma [96]? Might an inflammatory environment, particularly that induced by a high EBV load in IM, allow rogue T cell priming, leading in one case to cross-reactive auto-immune recognition of neuronal cells and in the other to wrongly polarized T cells that nurture rather than deter lymphoma growth? At the same time, the immune response can be used for good rather than ill; adoptive T cell therapy has shown how a restoration of virus-specific T cells can successfully treat EBV-driven B-lymphoproliferative disease [56; **Bollard et al, 2012**], so how best to exploit these same cellular responses to target other EBV-positive malignancies [97]? Arguably most important of all, how best to design a prophylactic vaccine that, even if it cannot prevent primary infection, may limit virus load and protect against EBV-associated disease [98]? As we approach the 50th anniversary of the discovery of EBV [99], the virus' challenge to the immunologist remains as potent as ever.

Figure legends

Figure 1.

A schematic view of EBV infection and persistence in the immunocompetent host. Orally transmitted virus establishes a lytic (Lyt) infection of permissive cells in the oropharynx , leading to high levels of virus shedding in saliva/throat washings. It is thought that both squamous epithelial cells and locally-infiltrating B cells may support this lytic infection; whether de novo-infected B cells can enter lytic cycle immediately or only after a phase of growth transformation is not known. Thereafter the virus colonises the general B cell system via growth-transforming latent infections of B cells in local lymphoid tissues such as the tonsil; denoted L3, these cells exhibit "latency 3" infection like that seen in LCL cells in vitro. Some L3 cells then down-regulate the expression of growth-transforming proteins, thereby escaping immune recognition and establishing a true antigen-negative form of latency (L0, "latency 0"); such cells are found exclusively in the memory B cell pool and re-circulate predominantly between the blood and oropharyngeal lymphoid tissues. On occasions, perhaps induced by cognate antigen-driven differentiation to plasma cells or by local mucosal signals, latently-infected cells within the B cell reservoir reactivate into lytic cycle. The virions thus produced can then seed low-level foci of replication/shedding in oropharyngeal epithelial cells, or initiate a new cycle of infection and transformation in adjacent B cells, some of which may survive to enter the memory B cell pool as above. In the immunocompetent host, many of these events are subject to immune controls. Primary EBV infection, as seen in IM patients, induces NK cell activation, large expansions of virus-specific $CD8^+$ T cells (with lytic antigen reactivities dominant over latent) and smaller expansions of virus- specific $CD4^+$ T cells (with latent antigen reactivities at least the equal of lytic) in the blood. These initial expansions are later culled, leaving lower numbers of virus-specific memory T cells in the blood of long-term virus carriers. Note, however, that the blood picture is not always representative of the situation in lymphoid tissues. Thus, the percentage of EBV-specific T cells in IM blood over-estimates that seen in tonsils during acute infection, whereas the situation is reversed in long-term carriers where EBV-specific memory populations, particularly latent antigen-specific $CD8^+$ T cells, are enriched in tonsils. Red arrows denote transfer of infectious virions; black arrows denote movement/transition of infected cells; blue arrows denote targeting of infected cells by cell-mediated immune responses.

Figure 2

Dynamics of virus load and immune response to primary symptomatic EBV infection. IM patients develop symptoms approximately 4-6 weeks after virus acquisition and this prodromal period is indicated as a shaded area on the graphs; dotted lines within this area indicate uncertainty about when parameters rise during this period. Virus genome loads : By the time IM

symptoms appear, high loads of infectious virus are being shed from sites of virus replication in the oropharynx, and high numbers of latently-infected B cells are detectable in the blood. While latently-infected B cell numbers fall rapidly over the course of disease, virus shedding in the throat typically remains high for several months before decreasing to lower levels. Cell-mediated responses : NK cell numbers are transiently raised in IM blood and the cells display an activated phenotype. At the same time, large numbers of activated lytic antigen-specific $CD8^+$ T cells dominate the blood picture, with the response to an individual immunodominant epitope often accounting for 5-25% or more of the expanded $CD8^+$ T cell population. These numbers fall rapidly as the disease resolves. Latent antigen-specific CD8 responses are less numerous and less heavily culled. Primary $CD4^+$ T cell responses are also detectable in IM blood but, by comparison, are much smaller and even more rapidly culled; individual epitope responses rarely exceed 1% of the $CD4^+$ T cell pool, with individual latent antigen reactivities dominant over lytic. Note that, unlike responses to all other target antigens studied, the $CD4^+$ T cell response to EBNA1 does not appear until weeks/months after IM. Antibody responses : By the time IM symptoms appear, IgG antibody titres to lytic cycle antigens such as the virus capsid antigen (VCA) complex have already peaked and later fall to stable levels. Likewise IgG antibodies to the EBNA2 latent cycle antigen are present but later fall, sometimes below the level of detection, whereas IgG responses to EBNA1 first appear months later but then persist for life. Thus an anti-VCA^+, anti-$EBNA2^+$, anti-$EBNA1^-$ serologic picture is diagnostic of primary/recent EBV infection, and full resolution of a primary infection is associated with the anti-EBNA1 antibody titre exceeding that of anti-EBNA2.

Figure 3

Antigen-presenting cells and EBV-specific T cell responses: Upper diagrams T cell responses may be driven by direct contact with virus-infected B cells themselves, or by viral antigens released from infected cells and cross-presented by dendritic cells. Note that all latent and lytic cycle proteins tested to date are detectably released from infected cells, with the single exception (not shown) of EBNA1. Lower diagrams Using $CD8^+$ T cells as an example, effective T cell recognition of B cells requires both TCR ligation of the cognate HLA I-peptide complex and licensing by SAP-dependent CD244/CD48 and NTBA/NTBA interactions; this licensing fails in XLP patients. Dendritic cells do not express CD48 or NTBA and so their recognition by T cells is independent of these additional licensing interactions.

[A] In the immunocompetent host: Two features of EBV-induced $CD4^+$ T cell responses, the broad spectrum of antigens targeted (including many late lytic cycle proteins) and the delayed primary response to EBNA1 (a poorly-released antigen), both suggest a dominant role for antigen release and cross-presentation as the main driver of these responses; it seems likely that dendritic cells play a key role in such cross-presentation, although EBV-infected B cells can also acquire viral antigens released from infected neighbours and cross-present to $CD4^+$ T cells.

Conversely two features of the EBV-induced $CD8^+$ T cell response, the marked focus on epitopes from immediate early and some early proteins of the lytic cycle (reflecting epitope display on naturally-infected cells) and the rapid primary response to EBNA1 (a poorly-released antigen), both suggest that the CD8 response is predominantly driven by direct contact with infected B cells.

[B] In XLP patients : In the absence of SAP, T cell/B cell recognition is impaired. This should not affect the antigenic profile of $CD4^+$ T cell responses since the main cell-cell interaction driving these responses, $CD4^+$ T cells and cross-presenting dendritic cells, should be intact. However, $CD8^+$ T cell responses may now also become dependent upon the cross-presenting pathway; as a result, the antigen profile of the CD8 response is predicted to be broader and no longer reflect the hierarchy of epitope display on naturally infected B cells. Note that the failure of T cell/B cell recognition in XLP also impairs receipt of the TCR-restimulation signals that normally regulate $CD8^+$ T cell expansions, thereby promoting hyper-expansion of the response and its attendant immune pathology.

Figure 4.

The humanised mouse model of EBV infection : NK cells restrict lytic EBV replication to prevent IM symptoms. [A] NOD/scid/$\gamma c^{-/-}$ mice are reconstituted with human immune system components using human $CD34^+$ haemopoietic stem cells and then experimentally infected with EBV [B] Depletion of $CD94^+NKG2A^+KIR^-$ NK cells does not affect levels of growth-transformed latently-infected cells (L3) but abrogates control of lytic EBV replication, leading to higher numbers of lytically-infected cells (Lyt) in lymphoid tissues. This in turns drives increased large $CD8^+$ T cell expansions, associated with IM-like symptoms. In contrast, animals retaining NK cells are able to control lytic EBV infection and thereby avoid this lytic antigen-specific $CD8^+$ T cell expansion.

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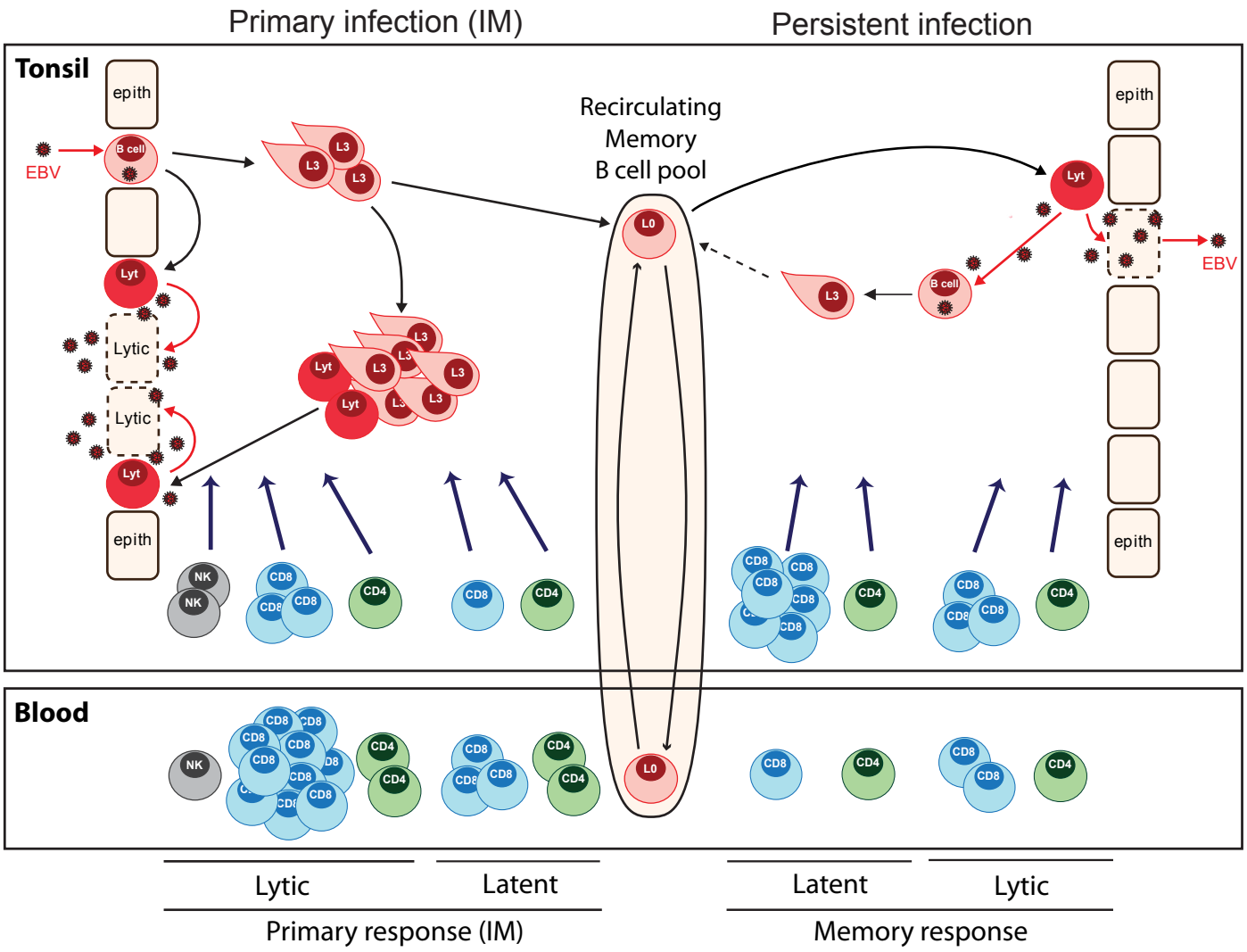
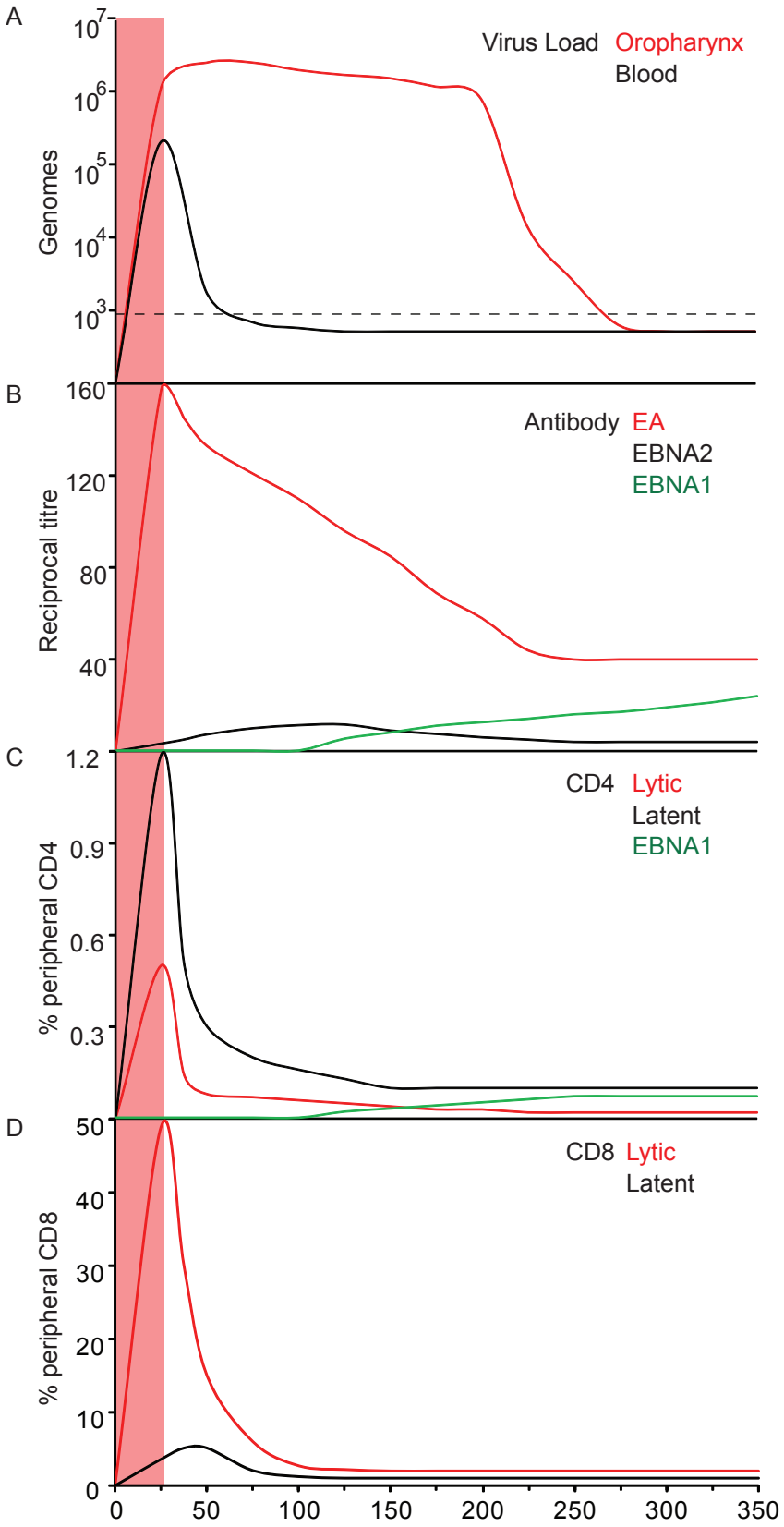

Primary infection (IM)
Persistent infection
Tonsil
epith
B cell
EBV
L3
Lyt
Lytic
Recirculating
Memory
B cell pool
L0
NK
CD8
CD4
Blood
Lytic
Latent
Primary response (IM)
Memory response

Figure 1.


A
Virus Load
Oropharynx
Blood
Genomes
10^7
10^6
10^5
10^4
10^3
B
Antibody
EA
EBNA2
EBNA1
Reciprocal titre
160
120
80
40
C
CD4
Lytic
Latent
EBNA1
% peripheral CD4
1.2
0.9
0.6
0.3
D
CD8
Lytic
Latent
% peripheral CD8
50
40
30
20
10
0
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100
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200
250
300
350

Figure 2

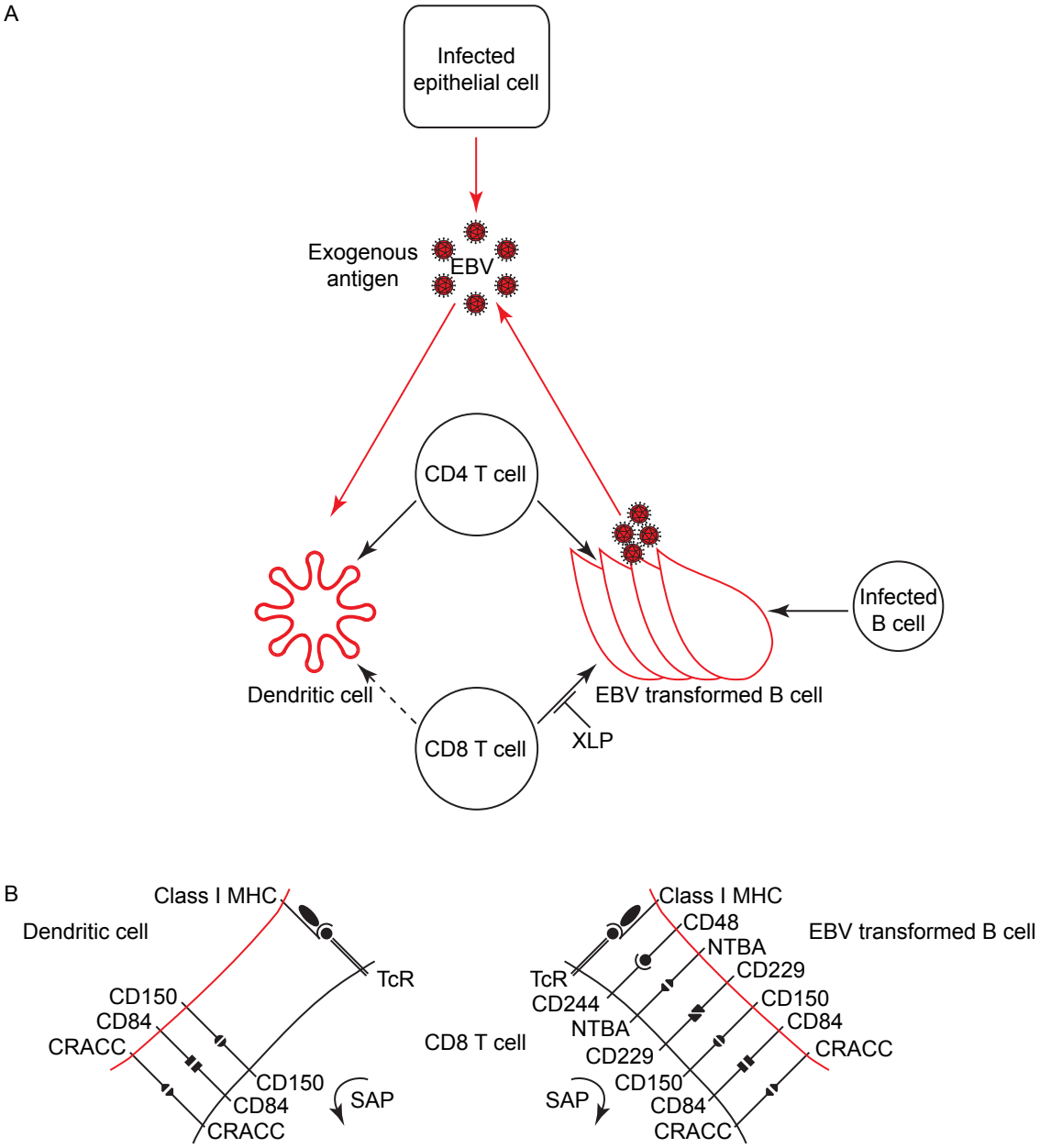
A
Infected epithelial cell
Exogenous antigen
EBV
CD4 T cell
Infected B cell
Dendritic cell
EBV transformed B cell
CD8 T cell
XLP
B
Class I MHC
Dendritic cell
TcR
CD150
CD84
CRACC
CD150
CD84
CRACC
SAP
CD8 T cell
Class I MHC
CD48
EBV transformed B cell
NTBA
TcR
CD229
CD244
CD150
NTBA
CD84
CD229
CRACC
CD150
SAP
CD84
CRACC

Figure 3

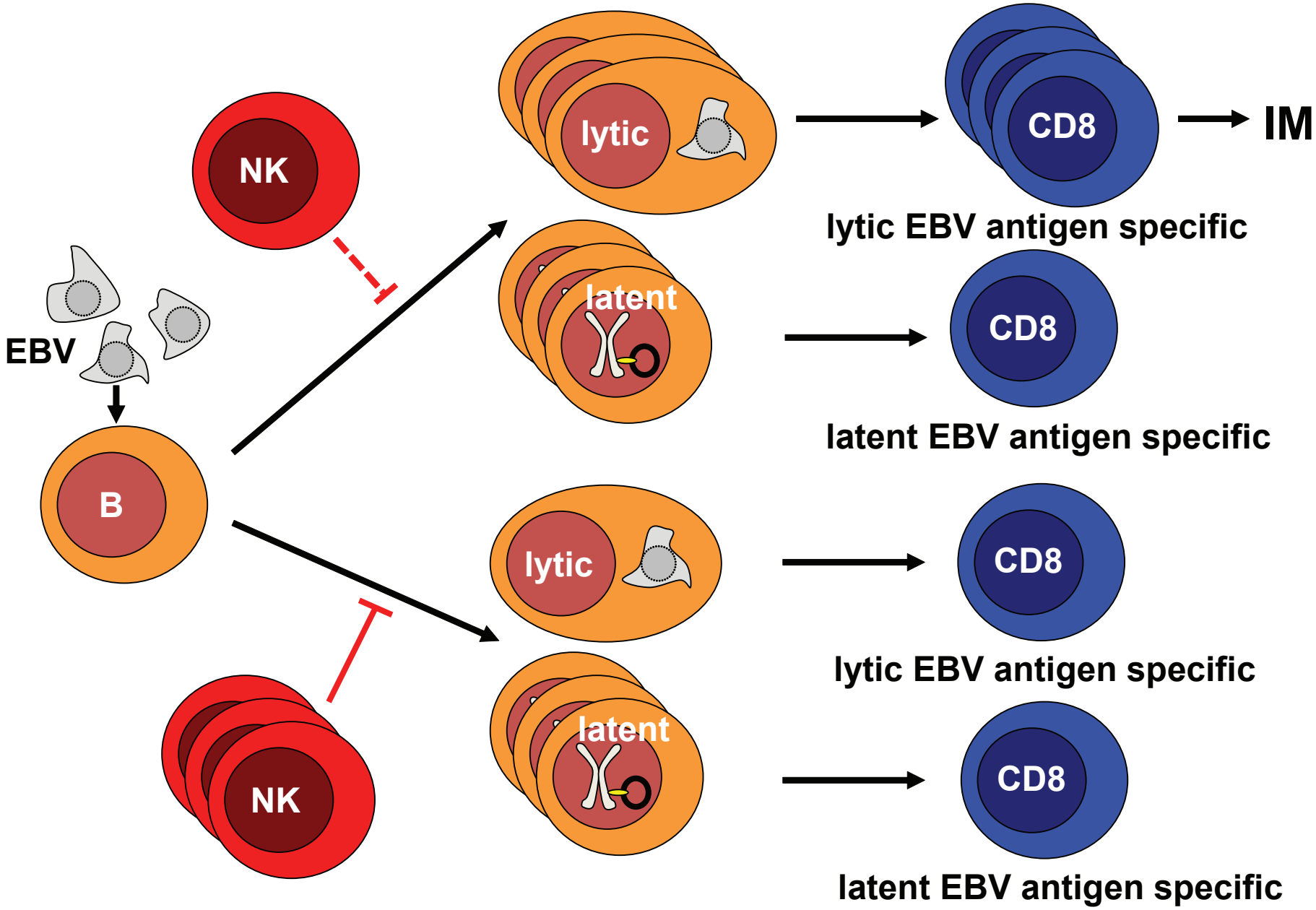
NK
EBV
B
lytic
latent
CD8
IM
lytic EBV antigen specific
CD8
latent EBV antigen specific
lytic
CD8
lytic EBV antigen specific
NK
latent
CD8
latent EBV antigen specific

Figure 4